The expression of plant vicilin DNA in yeast

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FIG.3.27

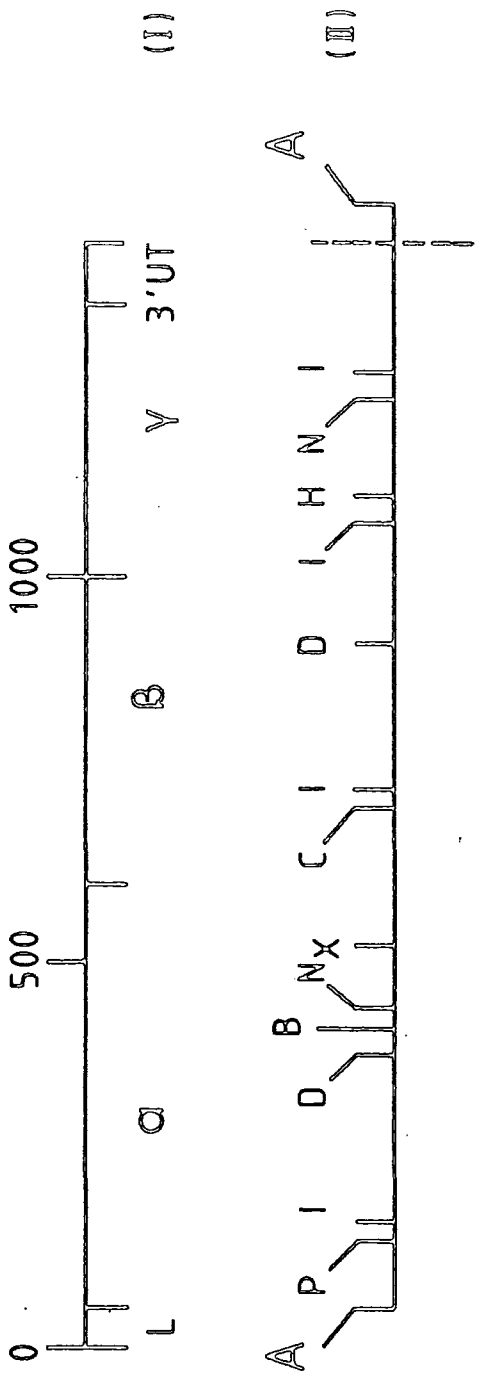


FIGURE 3.2.7

(i) The idealised vicilin cDNA message showing α, β, and γ subunits, the leader sequence (L), and the 3' untranslated region. The numbers represent the size scale in bp.

(ii) The vicilin cDNA (restriction map) used in the project - no leader sequence. This corresponds to that in plasmid pDUB9, i.e. 50K typeC vicilin. A = BamHI, B = BglII, C = AccI, D = HincII, H = HindIII, I = HinfI, N = BstNI, P = PstI, and X = XbaI.

vector was cloned as a partial EcoRI fragment from the plasmid YEp213-12 which was derived from the standard vector YEp213 by filling-in of the HindIII site in the 2μm DNA. The bacterial sequences (SalI-EcoRI) were from pBR328 (i.e. Ap^R, Tet^S, and Cm^S). The *CYC1* terminator DNA was derived from a HaeIII-HindIII fragment with the HindIII end filled-in. The HaeIII end is proximal to the polylinker HindIII sites of the vector. Except for EcoRI, the polylinker sites labelled are unque in the plasmid. Therefore this Yeast secretion vector is based on *MFα1*.

It was envisaged the cDNA vicilin (Figure 3.2.7(ii)- Type C, 50K, no glycoslyation sites, but has two proteolytic cleavage signals at the α-β and β-γ subunit junctions and hence gives rise to the Mr 12,500, 13,500, and 19,000 subunits (Figure 3.2.7(i)), would be inserted downstream of the *MFα1* gene prepro sequence allowing expression and secretion of the functional vicilin protein. The prepro sequence being removed proteolytically by the yeast. This would obviously depend on the extent of base pair deletion, and therefore the frame shift imposed in the recombinant plasmids pL2 to 6. The plasmid pL1 having no deletion imposed, so that a comparison could be made to the Δ plasmids.

The expression would therefore be under the control of the α mating factor, and the sequences in the cDNA. A transcription termination sequence is down stream of the vicilin cDNA, the 280 bp *CYC1* terminator. So the expression is dependant on a number of factors in the vector and cDNA, the only ones not constant in all the plasmids pL1→6 is the amount of deletion, and therefore the frame shift imposed.

The mating type of the cell controls the secretion of the α mating factor protein produced by the *MFα1* gene which is only expressed in either α or a or a/α diploid cells. Mackay and Manny (1974)[96] proposed that the mating type loci code for regulatory proteins that control expression of other genes coding for mating- and sporulation-specific processes. This locus has at least two complementation groups, called *MATα1* and *MATα2*. Each haploid cell type secretes into the culture medium a specific oligopeptide pheromone, or mating factor. These extracellular signals trigger biochemical changes reciprocally in their respective target cells: haploids of the opposite mating type. The responses elicited include, in order of their appearance, (1) cell-surface alterations that enhance the strength and selectivity of cell-cell contacts; (2) transient arrest of cell growth, specifically in G_1 phase of the cell cycle, that stays nuclear DNA synthesis and, effectively, synchronises the growth stages of the mating partners; and (3) new wall and membrane synthesis that is appropriate to cell fusion rather than to normal budding. Heterozygous diploids (a/α cells) neither produce pheromones nor do they respond to them.

The most attractive current picture for the function of the *MAT*a and *MATα* loci is that their specific transcripts encode separate sets of regulatory proteins [96,50,90,92]. The regulatory proteins encoded by a particular *MAT* locus are essential for controlling the expression of other genes elsewhere in the yeast genome, which determine many of the cell's characteristics (its mating ability, its budding pattern, its capacity for sporulation, etc.). Such a model would predict that a and α haploids differ detectably in the types of poly(A)-containing mRNA sequences that they express, althoughg this has not yet been

demonstrated directly, except for the transcripts from the *MAT* regions themselves. Of special interest here is that particular genes at *MAT* direct whether or not a cell will elaborate a mating pheromone (and if it does so, which pheromone will it produce).

The vicilin cDNA used in this experiment (figure 3.2.7) has no leader sequences, but contains the α, β, and γ subunits and the 3' untranslated (UT) region. By using a full length vicilin cDNA with a leader sequence the vicilin would be expressed and secreted into the vacuole, this can be compared to the secretion of the enzyme carboxypeptidase Y, which is secreted into the vacuole; whereas the α mating factor or the enzyme invertase is secreted into the plasma membrane and cell wall. The secretory pathway, in yeast, is well documented [92,80,68,69,67,95,41,26,25] (see section 1.6), and the process of vicilin through this pathway could be further elucidated using the *sec* mutants, obtainable from Dr. Randy Schekman, who determined, using these mutants, the yeast secretory pathway. The pathway is shown in figure 3.4.6, this can be compared to that found in higher eukaryotes (figure 3.4.7).

Although not all of this part of the project was achieved, the recombinant plasmids pL1 to 6 were made, but the time span of the project was not enough to finish the rest of the project.

3.3 YEAST TRANSFORMATION.

3.3.1 Aims and Strategy.

The aim of this part of the project was to study the various types of Yeast transformations systems used in section 3.1 and 3.2, comparing the yields and ease of manipulations, in *S. cerevisiae*.

3.3.2 Yeast transformation.

3.3.2.1 LiAc transformation.

Section 2.2.14.1 shows the method used to transform the yeast. The results are shown below in table 3.3.1. The number of cells/ml were determined from a graph obtained from growth curve experiments (figure 3.3.1), for all transformation experiments ($O.D._{600}$).

TABLE 3.3.1 Results of the LiAc transformation.

yeast strain	plasmid type	Transformants/µg DNA
MT302/1c	pMA257	$3.4 \pm 0.5 \times 10^5$
	pDUB2017/2018	$3.9 \pm 0.6 \times 10^4$
MD40/4c	pMA257	$1.6 \pm 0.4 \times 10^5$

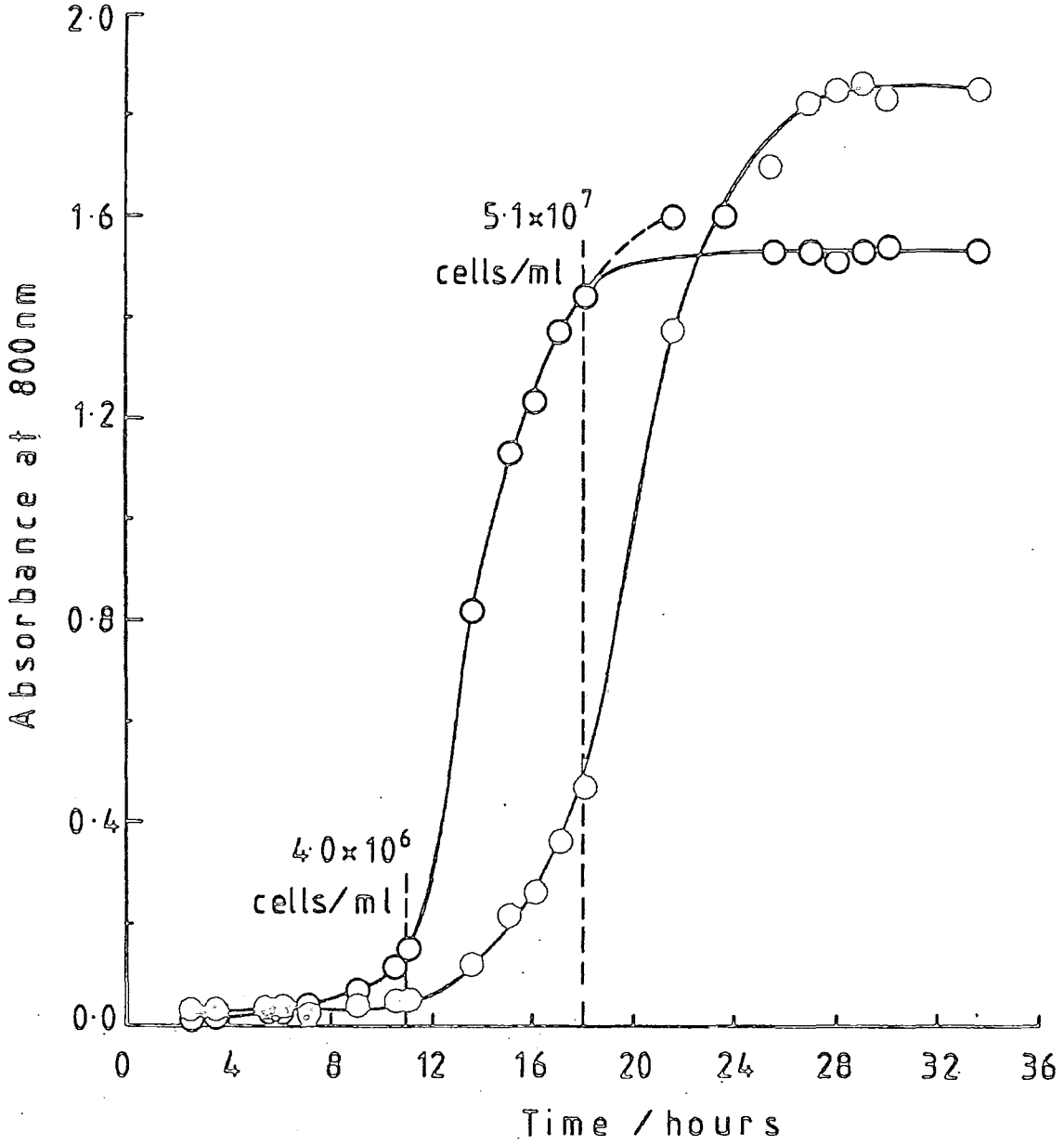

2·0
1·6
1·2
0·8
0·4
0·0
Absorbance at 800nm
5·1×10^7
cells/ml
4·0×10^6
cells/ml
0
4
8
12
16
20
24
28
32
36
Time / hours

FIG.3.3.1

FIGURE 3.3.1

The growth curves of *S. cerevisiae* (o), and *S. pombe* (o). The numbers (cells/ml) given are only for *S. cerevisiae* - early and late log. phase. This allows an estimate of the number of cells/ml in a particular culture.

3.3.2.2 Protoplast Transformation.

Section 2.2.14.2 shows the method used to transform the yeast. The results are shown in table 3.3.2

TABLE 3.3.2 Results of the protoplast transformation.

Enzyme	yeast strain	plasmid type	Transformants/µg DNA
Novozyme SP234	MT302/1c	pMA257	$6.6 \pm 0.6 \times 10^{3}$
		pDUB2017/2018	$5.0 \pm 0.5 \times 10^{3}$
Lyticase	MD40/4c	pMA257	$1.9 \pm 0.6 \times 10^{4}$
Glusulase	MT302/1c	pMA257	$3.0 \pm 0.8 \times 10^{5}$
		pDUB2017/2018	$1.4 \pm 0.4 \times 10^{5}$

3.3.3 Discussion.

These results show that the transformation rates (transformants/µg DNA) are quite favourable, in the order of 10^{3-5}, and only tend to vary due to the plasmid or enzyme used.

The LiAc transformation system shows the rates vary only due to the type of plasmid used, i.e. the plasmid pMA257 = $3.4 \pm 0.5 \times 10^{5}$ and plasmid pDUB2017/2018 = $3.9 \pm 0.6 \times 10^{4}$ (the results for both pDUB2017 and 2018 are

summated and the mean figure is shown, as they are the same plasmid - see below), this shows a difference of the order 10^1 for the *S. cervisiae-MT302/1c* strain. The strain type does not seem to determine to a great extent the rate of transformation, i.e. the strain MD40/4c = 1.6 ± 0.4 x 10^5 for the plasmid pMA257, compared to the same plasmid transforming the strain MT302/1c (3.4 ± 0.5 x 10^5), the later therefore is transformed approximately two x greater than the MD40/1c strain for the same plasmid. The differences observed between the plasmids pMA257 and pDUB2017 may be explained due to the difference in size. The plasmid pDUB2017/2018 is the product of the pMA257 plus a cDNA vicilin fragment of 1.43 Kb (2017 has the cDNA in the wrong oriention, 2018 the correct orientation). The stearic hinderence involved may account for the phenomenon, the smaller the DNA the greater the ease of entering the cell, and therefore the greater chance that the cell will be transformed to a greater extent.

These results are quite encouraging as the results obtained by Ito *et al.* (1983)[43] show that plasmids YRp7 and YRp6 transformed into *S. cerevisiae* strain AH-22 give 4 x 10^2 transformants/µg DNA. (Compared with 3.7 ± 1.6 x 10^3 transformants/µg DNA for the plasmid YRp7 in the strain MD40/4c - this result is not shown).

The protoplasting results show a similar phenomenon to that shown above between the sizes of the plasmids, but further to this the results show differences in transformation rate due to the type of protoplasting enzyme used. For the plasmid pMA257 the rates show a clear series for the enzymes used, i.e.

GLUSULASE > LYTICASE > NOVOZYME SP234

This seems to be true when viewing the results of plasmid pDUB2017 in these enzyme. In all cases the differences shows an order of 10^1 between each consecutive enzyme. This may be due a number of reasons; (i) the results may be a good observation, (ii) the results may be due the time span to which the cell where given in the enzymes - this sould not be the case as the time span were approximately the same in all cases, (iii) the amount of enzyme added to the reaction mixture - the experiment attempted to keep the same working concentration in each case, so this should not be a problem, (iv) it more likely that the differences may be due to the efficiency of each enzyme. Although the extent of protoplast formation was about the same with each enzyme.

Table 3.3.3 below shows the estimated transformation rates for particular types of plasmid transformed via protoplasting [72,74,106].

TABLE 3.3.3 Properties and transformation rates for yeast vectors.

Vector	Transformation rate/ Transformates/µg DNA	Loss in non-selective medium
YIp	1-10	<< 1% per generation
YEp	10^{3-5}	1% per generation
YRp	10^{2-3}	>> 1% per generation, but can not get chromosomal integration
YCp	10^{2-3}	< 1% per generation

Table 3.3.4 shows the advantages and disadvantages for each type of vector [72,74,106].

TABLE 3.3.4 Properties of yeast vectors.

Vector	Disadvantages	Advantages
YIp	(i) low transformation frequency (ii) can only be recovered from yeast by cutting chromosomal DNA with restriction endonuclease which does not cleave original vector containg cloned gene	(i) of all vectors this kind give most stable maintenance of cloned genes.(ii) an integrated YIp plasmid behaves as an ordinary genetic marker, e.g. a diploid segregates the plasmid in a Mendelian fashion. (iii) most useful for surrogate genetics of yeast, e.g. can be used to introduce deletions, inversions and transpositions.
YEp	(i) novel recombinants generated *in vivo* by recombination with endogenous 2μm plasmid.	(i) readily recoved from (ii) high copy number (iii) high transformaion frequency. (iv) very useful for complementation studies.
YRp	(i) instability of transformants	(i) readily recovered from yeast. (ii) high copy number - this is usually less than that of YEp vectors, but this may be useful if cloning gene whose product is deleterious to the cell if produced in excess. (iii) high transformation frequency. (iv) very useful for complementation studies. (v) can integrate into

		the chromosome.
YCp	(i) low copy number makes recovery from yeast more difficult than that YEp or YRp vectors.	(i) low copy number is useful if product of cloned gene is deleterious to cell. (ii) high transformation frequency. (iii) very useful for complementation studies. (iv) at meiosis generally shows Mendelian segregation.

These tables above show that the transformation rates results are approximately in the same order (i.e. the YEp plasmids), so the results compare favourably.

The yeast *S. pombe* could be not successfully transformed, although the transformation procedures are similar for both *S. pombe* and *S. cerevisiae*, the former is more difficult to transform successfully (Dr. P. Nurse - personal communication). This procedure would require further time to acheive a working system.

Transformation of yeast with cloned yeast DNA containing a selectable marker was first accomplished by Hinnen et al. (1978)[45] and then Beggs (1978)[6,7]. The *LEU2* gene was used, which had already been isolated on the basis of its ability to function in *E.coli*, as a marker readily selectable in yeast. Having such a selectable marker is essential, since DNA transformation is (not surprisingly) a rare event. Two fundamentally different types of transformation

have been described by Struhl et al. (1979) [72,74,105]. These all described a variation on a theme, transformation with protoplasts prepared by lytic enzyme treatment. Recently a variety of attempts have been made to 'simplify' the procedure producing the required transformed cell, to get away from the necessity of protoplast transformation. But the transformation efficiences have not yet reached those of the best protoplast systems [89].(See method for procedure).

An easy method of yeast (*S.cerevisiae*) transformation without the need of making protoplasts has been developed by Ito et al. (1983) [43]. Previously , Triton X-100, a non-ionic detergent, was shown to alter yeast cell membranes so that various extracellular mononucleotides were incorporated into cells. This detergent had no effect on the viability of yeast cells. These observations suggested that yeast cells treated with Triton X-100 or other detergents might take up plasmid DNA like *E.coli* cells treated with $CaCl_2$. Therefore Ito et al. (1983) [43] studied the uptake of plasmid DNAs by intact yeast cells treated with various agents such as detergents and metal ions. They found that the alkali metal ions such as Li^+, Na^+, K^+, Cs^+, and Rb^+ are effective for inducing competence in yeast cells. But the detergents used were not as effective in inducing competence. The technique is based on the finding that yeast cells treated with alkali cations particularily lithium chloride or acetate are able to uptake plasmid DNA such as YRp7 in the presence of polyethylene glycol (PEG). Transformation efficiency by this method is at the level of one tenth that of a conventional protoplast method. PEG is indispensable for the DNA uptake, and alkali cations appear to increase the transformation frequency [43,48]. The yeast

transformation method developed by Ito et al. (1983,1984) [43,45] has the following advantages over the current protoplast method;

(i) It is simple, easy, and time saving.

(ii) Transformsation efficiency is comparable with that of the protoplast method for YRp7 (*ars1*), although it is much less efficient for plasmids with a 2μm origin of replication.

(iii) Replica plating of colonies is possible because no regeneration agar is necessary.

(iv) The method is applicable to yeast cells that are resistant or sensitive to lytic enzymes.

(v) LiAc transformed cells do not rearrange recombinant plasmids so readily as protoplasts.

Neumann et al. (1982) [32,43,75] reported that uptake of plasmid DNA into mouse lyoma cells was increased by electric field impluses (electroinjection or electroporation). Shivarova et al. (1983) [75] also reported that transformation frequency of *Bacillus cereus* protoplasts with plasmid DNA was increased by pulses in the presence of PEG. Recently, Hashimoto et al. (1985) [43], succeeded in the introduction of plasmid DNA into intact yeast cells by elecric field pulses and optimised electrical conditions. Interestingly, the maximum number of transformants (90±20/μg DNA) was obtained by three successive pulses with an initial intensity of 5 KV/cm and with a capacitance of 1 μF.

By the electroinjection method developed by Hashimoto et al. (1985) [43] as it know stands, the transformation frequency of intact yeast cells is somewhat

lower than that by the biochemical methods with alkali cations or thiol compounds [43,48]. Ito et al. (1983, 1984) [43,48] reported that the number of transformants per μg DNA of yeast cells with the aid of lithium is as high as 500-1300. The frequency of transformation by electroinjection may be improved by optimising non-electical experimental conditions such as concentrations of PEG or the presence of mono- and di-valent cations. Electroinjection is anovel method for transforming intact prokaryotic and eukaryotic cells with foriegn DNA.

In recent years, liposomes have been successfully used as vehicles for introducing genetic materials into cells. After encapuslation of RNA, chromosomes, and viruses into liposomes, the phospholipid membranes of the vesicles were induced to fuse with membranes of mammalian cells resulting in transfer of the liposome contents into the recipient cells [1,93]. With similar procedures tranfers of bacterial plasmids and plant viruses into plant cells; plasmids, chromosomal DNA, and phage DNA into bacterial cells have been demonstrated [1,93]. Ahn and Pack (1985) [1] extended the use of liposomes in transforming yeast cells. Although encapsulation of plasmids into liposomes did not increase the transformation efficiency, the liposomes protected plasmids from DNase action effectively. Further, as their data showed, once plasmids were encapsulated they could be transferred into yeast cells more efficiently compared to the naked plasmids.

3.4 ELECTRONMICROSCOPY OF YEAST.

3.4.1 Aims and Strategy.

The aim of this part of the project was to study and analyse, using electronmicroscopic techniques, the expression of the vicilin gene in yeast. The recombinant vectors from the previous sections, 3.1 and 3.2, were used and compared against the expression of the plasmid pDUB2018.

3.4.2 Electronmicroscopy.

The yeast *S. cerevisiae* was transformed, using the LiAc method (section 2.2.13.1), with the plasmid pDUB2018. Figure 3.2.6. shows a colony hybridisation, with a cDNA vicilin probe as used in section 3.2, to the pDUB2018 containing yeast cells. The transformed cells were then subjected to preparation (section 2.2.14.1) and immunogold gold labelling (section 2.2.14.2), prior to sectioning and examination of the labelled sections using a Transition Electron Microscope (TEM); the photographs taken are shown in figures 3.4.1 to 3.4.5. These results are discussed below (section 3.4.3). The transformants were not available from sections 3.1 and 3.2 due to the limited time span given to the project, and therefore this part of the project can not be acheived, but some predictions are possible from the results obtained using the plasmid pDUB2018.

3.4.3 Discussion.

FIGURES 3.4.1 to 3.4.5

These figures show micrographs of plasmid pDUB2018 transformed yeast at a variety of magnifications, using a 5 and 15nm (3.4.5 only) vicilin antibody label. (see text for details)

A

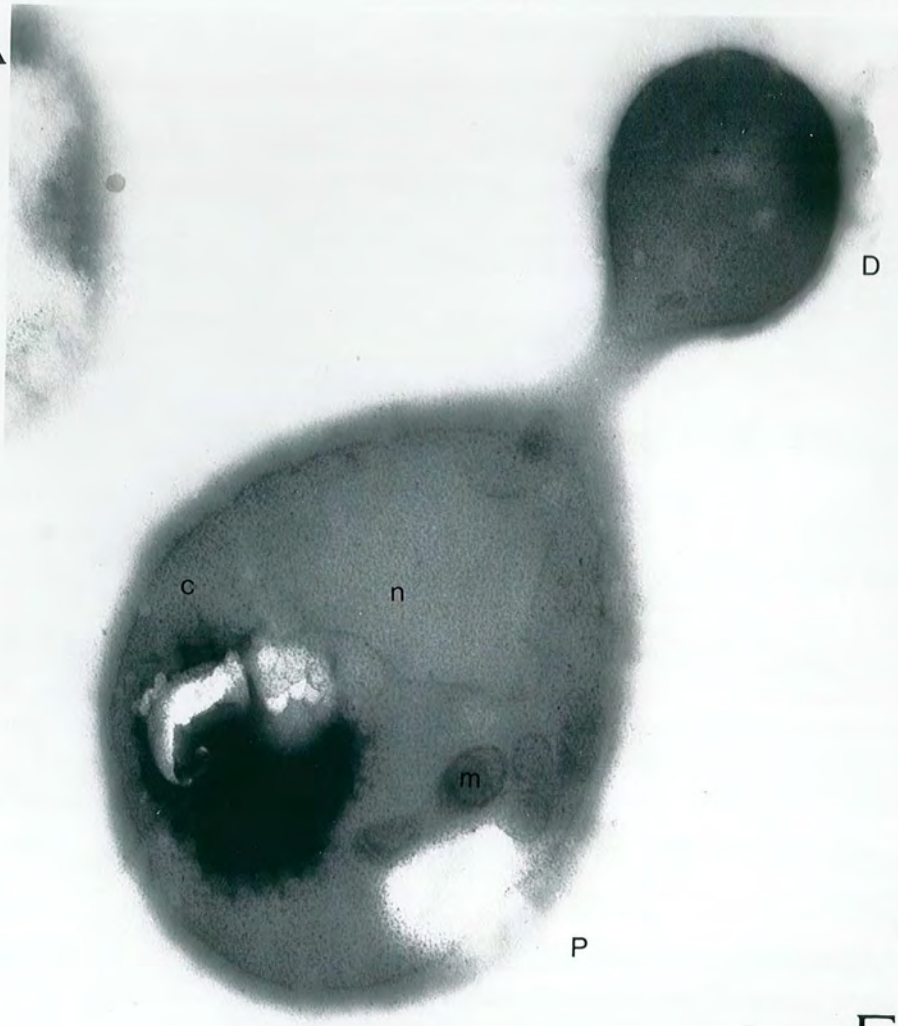
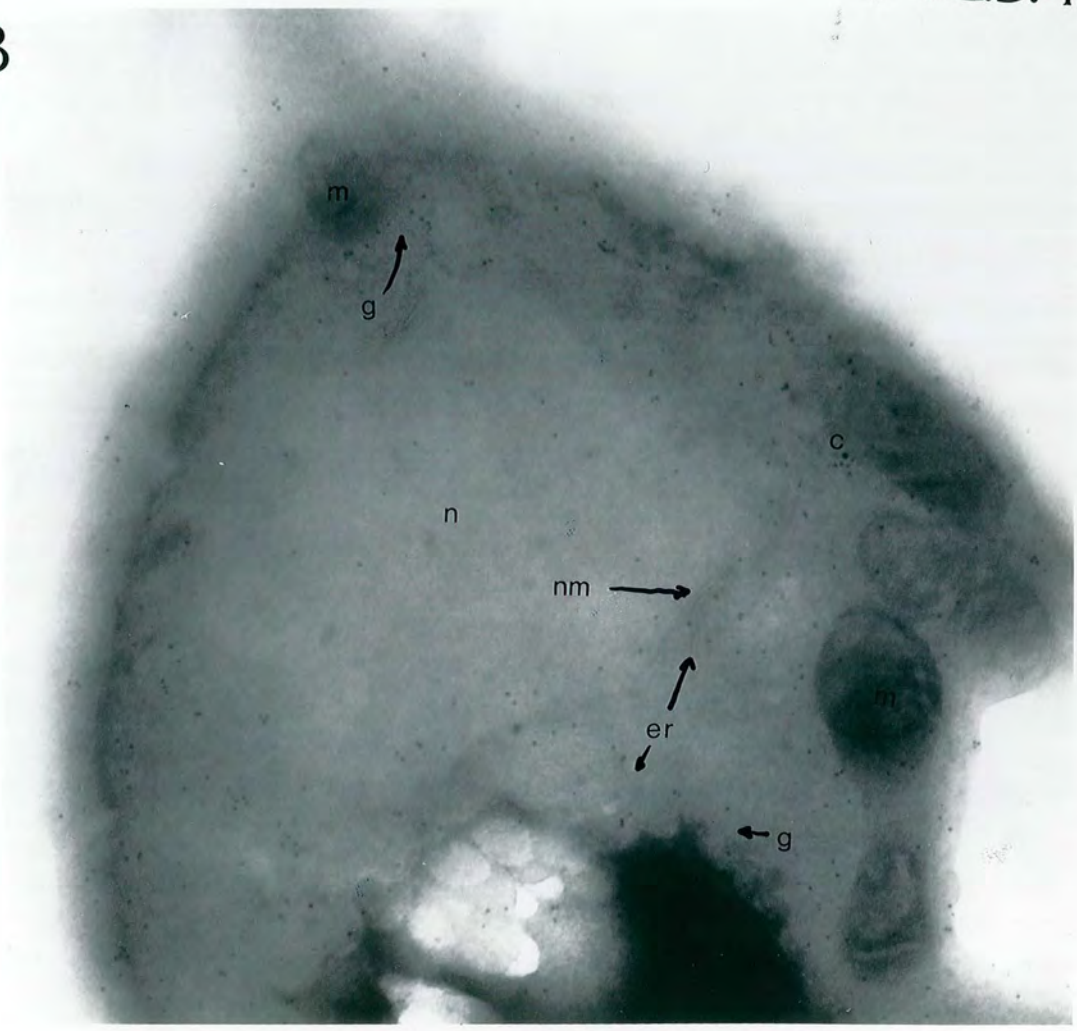


FIG.3.4.1

B



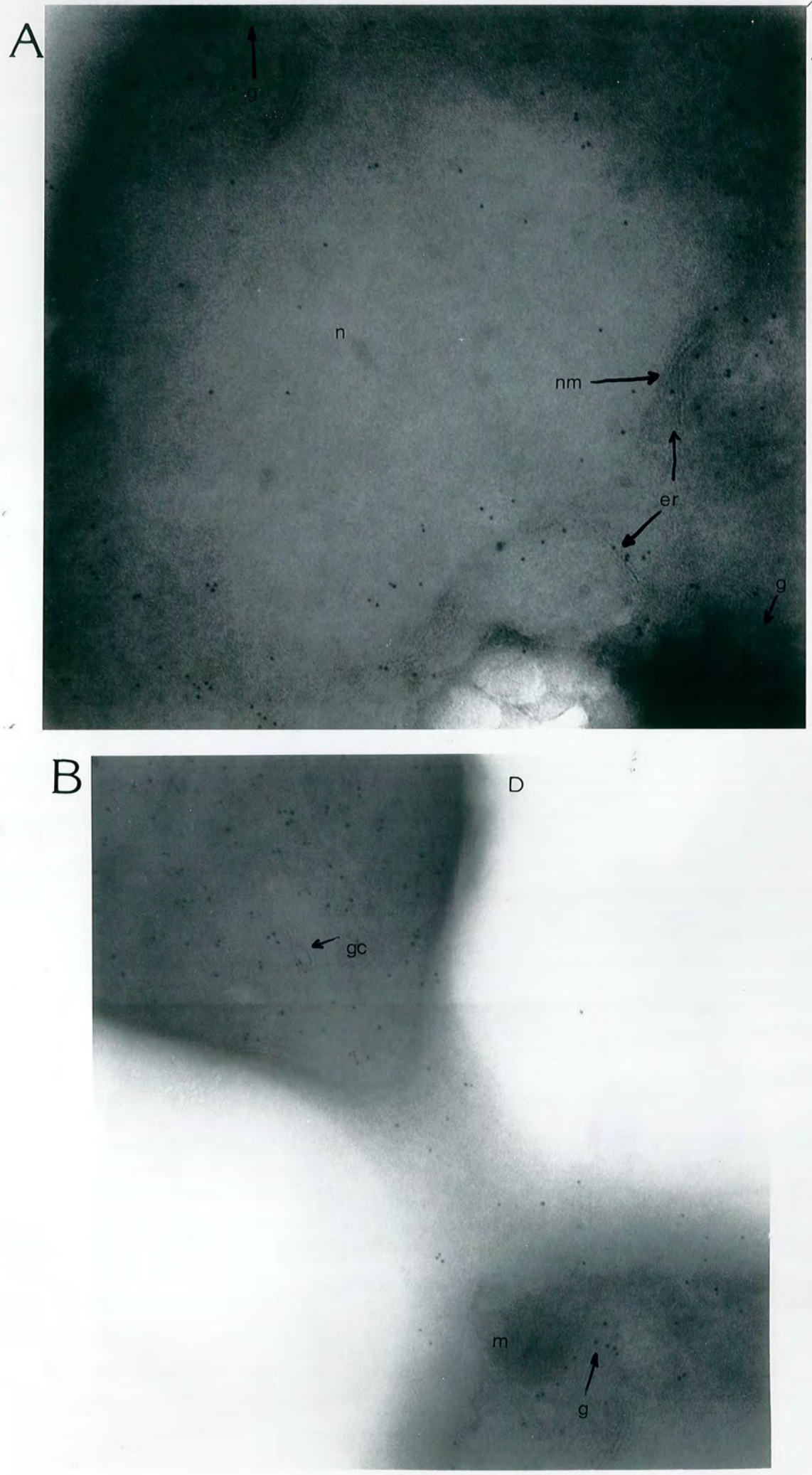


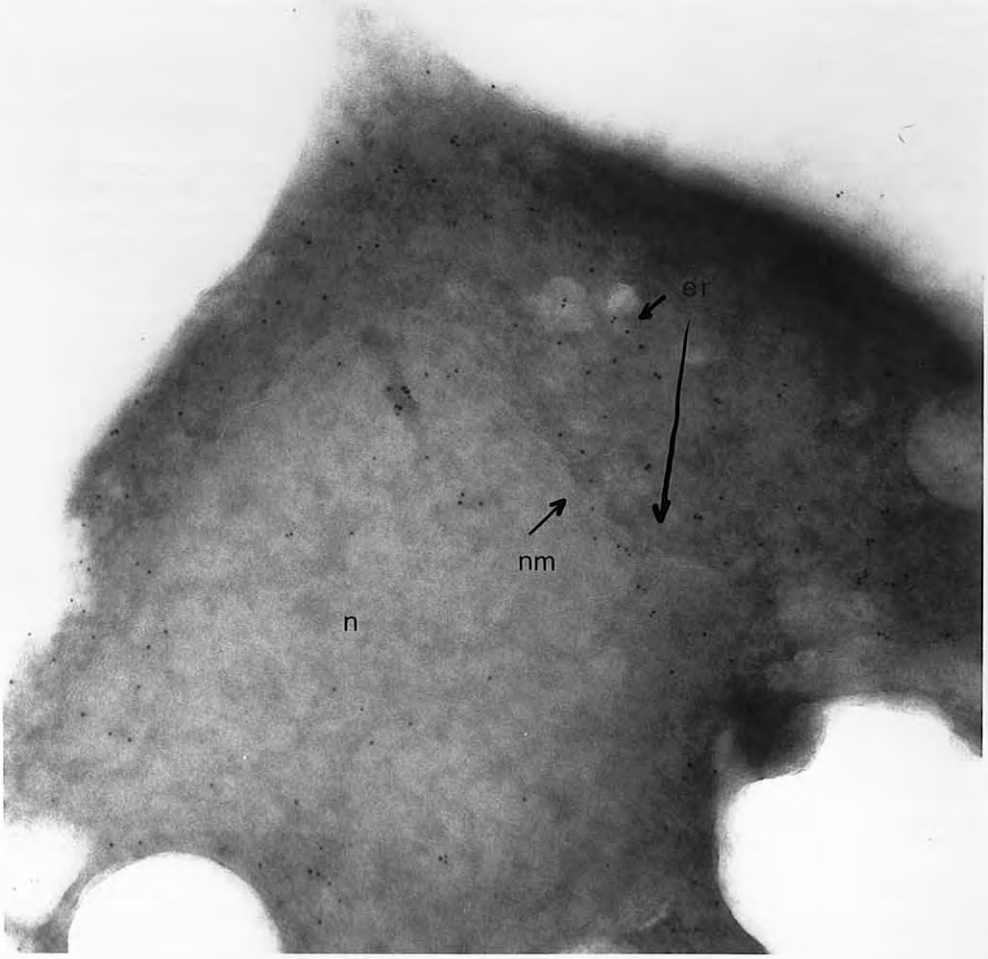
FIG.3.4.2

A

B

FIG3.43

A



B

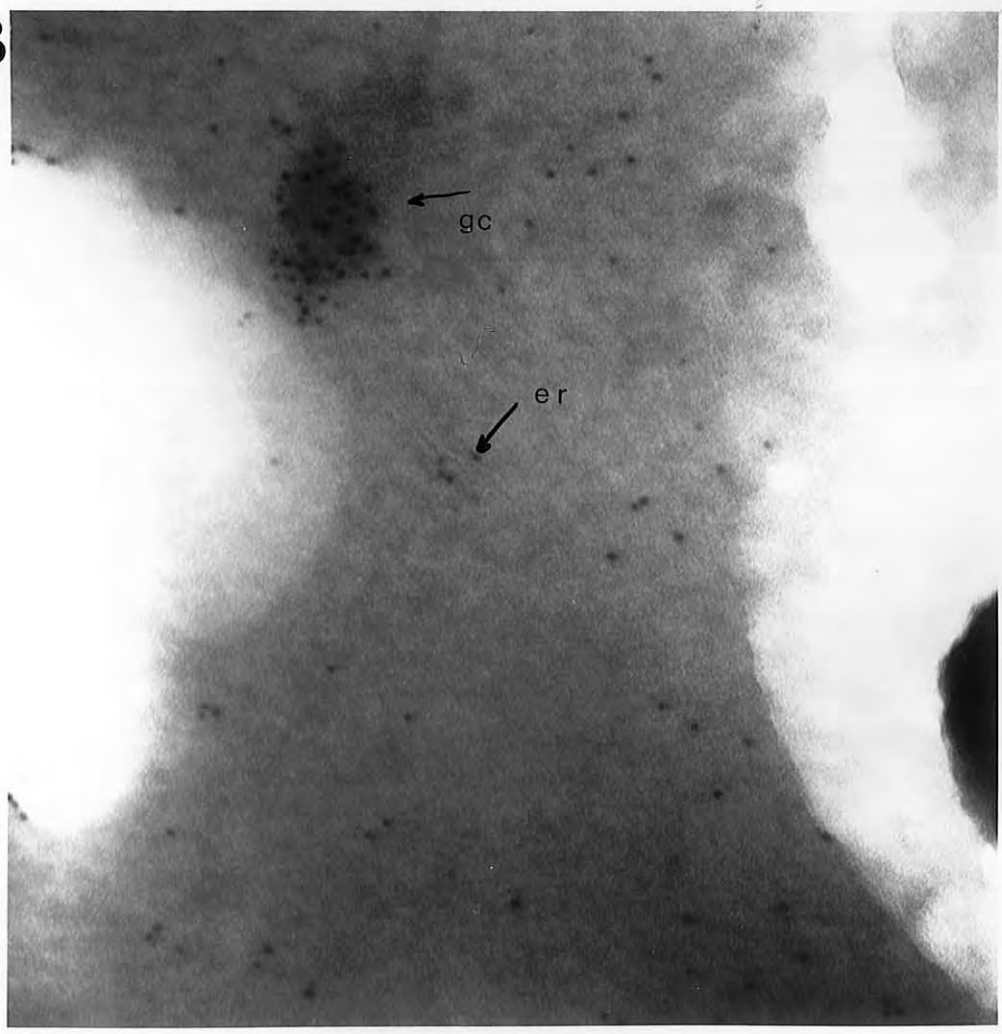


FIG.3.4.4

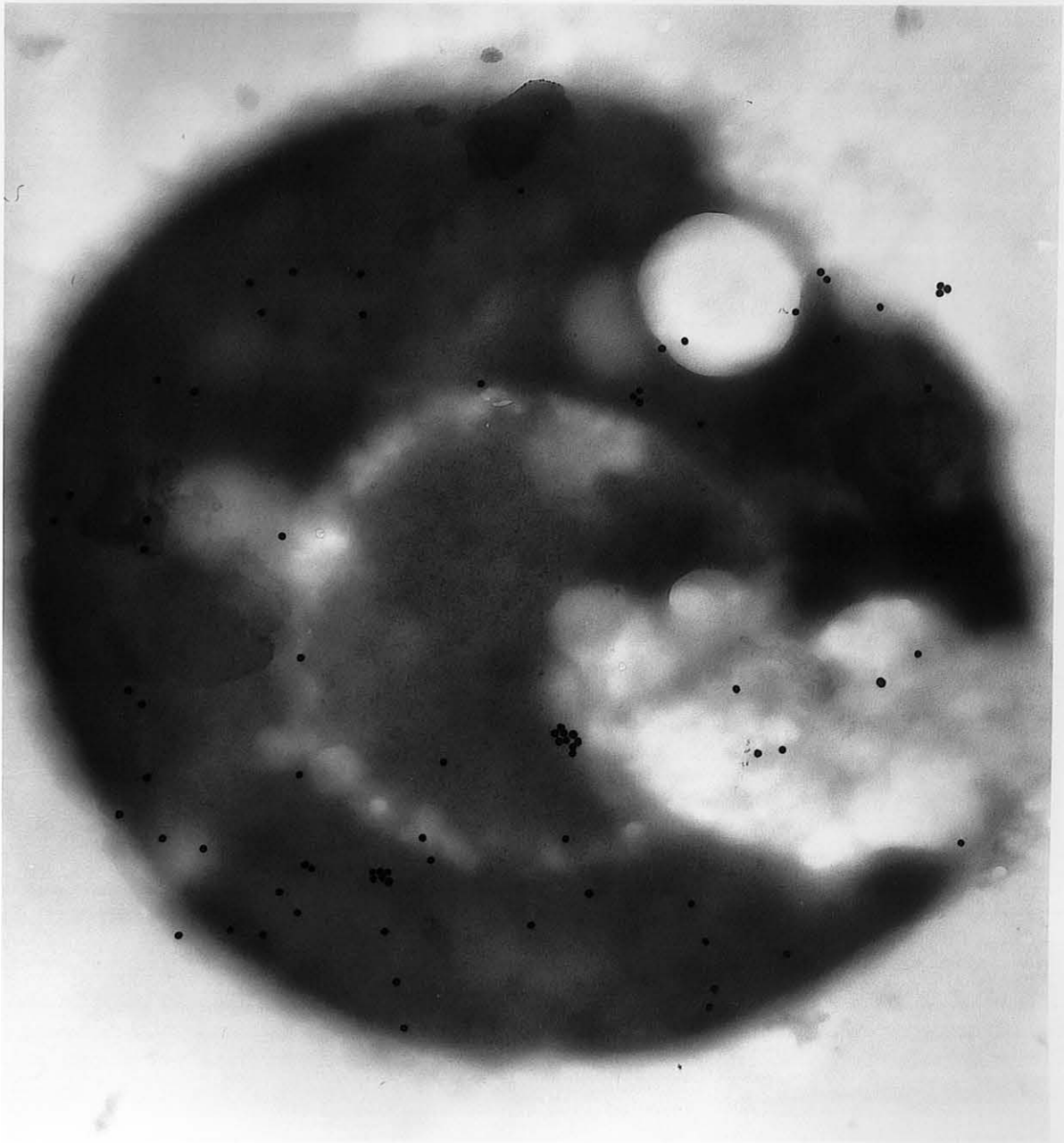


FIG.3.4.5

The plasmid pDUB2018 contains the cDNA vicilin insert (correct orientation) discussed in section 3.2. The plasmid itself is constructed from the plasmid pMA257 (A. J. Kingsman - unpublished), with a *LEU 2* region, which is defective; 2μ; pBR322; and a Phosphoglycerate Kinase (PGK) promoter, this contains only the 3' and 5' ends with a 14 amino acid region of the PGK N-terminus, and a single BamHI site between the 3' and 5' ends. This allows high copy number in the yeast, and therefore a large amount of the vicilin is produced - this can be observed in all the micrographs recorded (figures 3.4.1 to 3.4.5 using both the 5 and 15nm gold label).

The aim of this part of the project was to compare the results obtained from the other parts - sections 3.1 and 3.2, with the plasmid pDUB2018 results, this was not possible due to the time span of the project, and therefore only the pDUB2018 results can be discussed. It was hoped that the secretion pathway to the yeast vacuole could be shown (section 1.6 - figure 3.4.6 and 3.4.7).

Figure 3.4.1 A shows a budding cell of *S. cerevisiae* at the magnification x19,500. Various organelles and membrane structures can be seen, but at this magnification the gold labelling (black dots) are not easily discernable. Although this figure shows the overall view. Figure 3.4.1 B shows a more magnified view of this yeast, x43,000. The label (5nm) can mostly be observed in the cytoplasm (c), but can also be seen in the nucleus (n), and can be seen associated with the endoplasmic reticulum (er) and golgi apparatus (g).

Figure 3.4.2 A shows a greater magnified section of figure 3.4.1 (x70,000), this gives a clearer view of the nuclear region; showing black dots associated with the nuclear membrane (nm), the er, and the g. Moving up the section, figure 3.4.2 B (x70,000), shows the dots clearly membrane associated (g), and their association, in the daughter cell, with a possible golgi complex (gc) derived from the paternal cell.

Figure 3.4.3 A and B show daughter cells, B akin to the ones above (x90,000), and A with a magnification x70,000 akin to those in figure 3.4.4. B shows most of the cell; a variety of membrane structures with vicilin can be discerned, corresponding to er, gc and g. A shows putative membrane structures (↓), with possible associated vicilin. The vicilin dots seem to be associated with the darker staining cytoplasm, possibly vesicular.

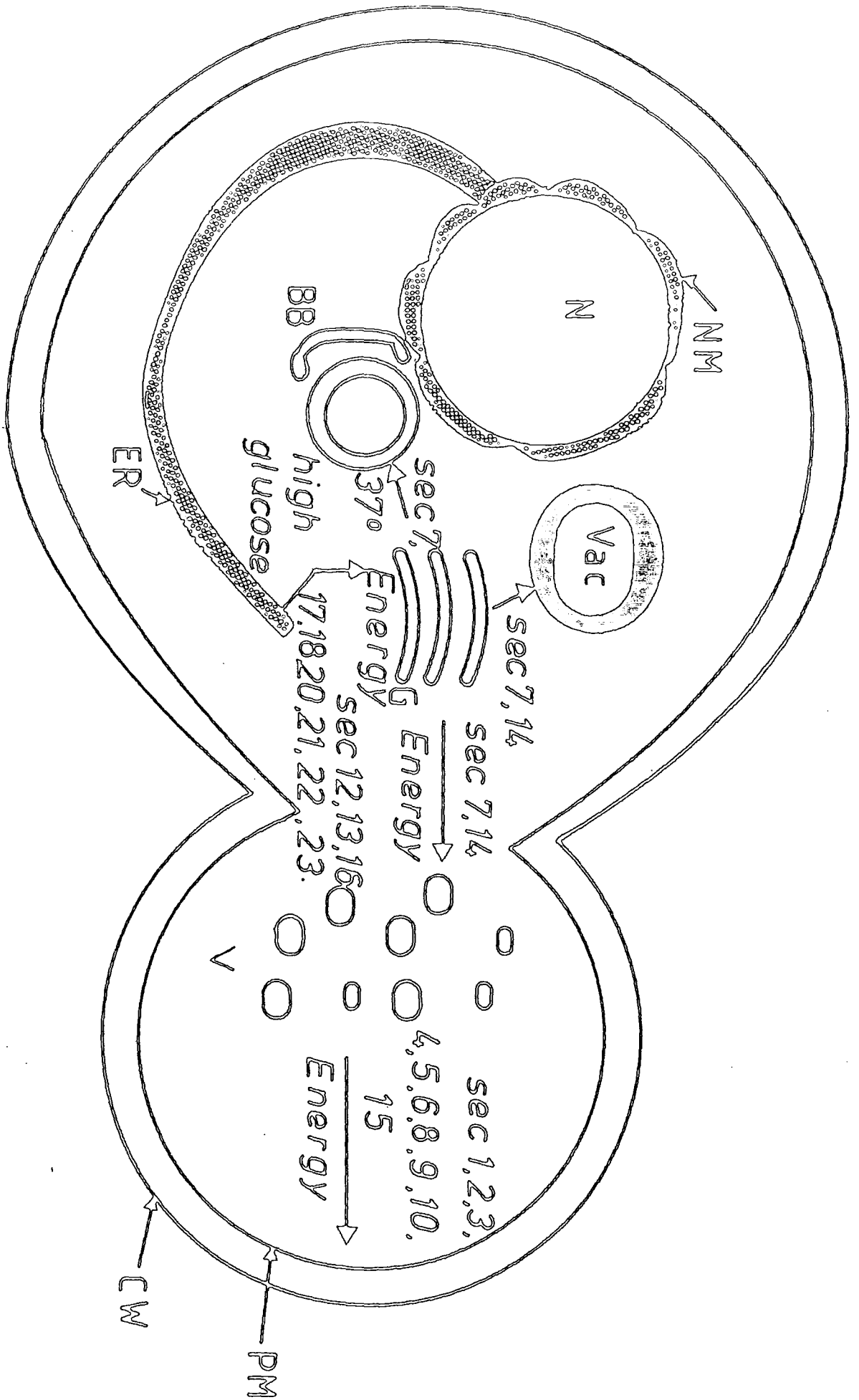
Figure 3.4.4 A shows a similar situation to figure 3.4.1 B, n, nm, and er. But figure 3.4.4 B shows a highly vicilin associated region, this is possibly vesicular (v) or gc in nature. This very important as the gc (Dr. G. Warren - personal communication), which have been reported in higher eukaryotes, may be present in yeast. Warren has proposed that these structures are an intermediate when the cell is duplicating itself. The golgi apparatus is disbanded into this intermediate, allowing movement throughout the cytoplasm, and reforming into the golgi again were required by the cell. It is possible that this structure, found on figure 3.4.4 B, is in the process of disbanding, possibly reappearing in such as the daughter cell. This could possibly show a unproposed form of transport in the yeast cell. Recently Dunphy *et al.* (1986) [25] have stated that

FIGURE 3.4.6

Secretory and Vacuolar protein transport pathways in yeast. The particular *sec* mutants are shown in each step of the pathway.

N = nucleus, NM = nuclear membrane, ER = endoplasmic reticulum, G = golgi apparatus, Vac = vacuole, BB = Berkley body, V = vesicles, PM = plasma membrane, and CW = cell wall.

FIG.3.4.6


NM
N
BB
high glucose
ER
37°
sec 7,
Energy
17,18,20,21,22,23.
sec 12,13,16
Energy
sec 7,14
VAC
sec 7,14
Energy
1,5,6,8,9,10,
15
sec 1,2,3,
LW
PM

yeast and mammals utilise similar cytosolic components to drive protein transport through the golgi apparatus, so it is therefore quite possible this intermediate complex well be found in yeast. Also the vicilin can be seen associated with a double membrane stucture, possibly er or g.

Figure 3.4.5 simply shows the difference between the 5nm, seen above, to the 15nm gold labelling. Although the section does not show organelle structure to any degree, it does show that the 5nm labelling is more specific and one is capable to discern a higher degree of compartmentalisation within the cells.

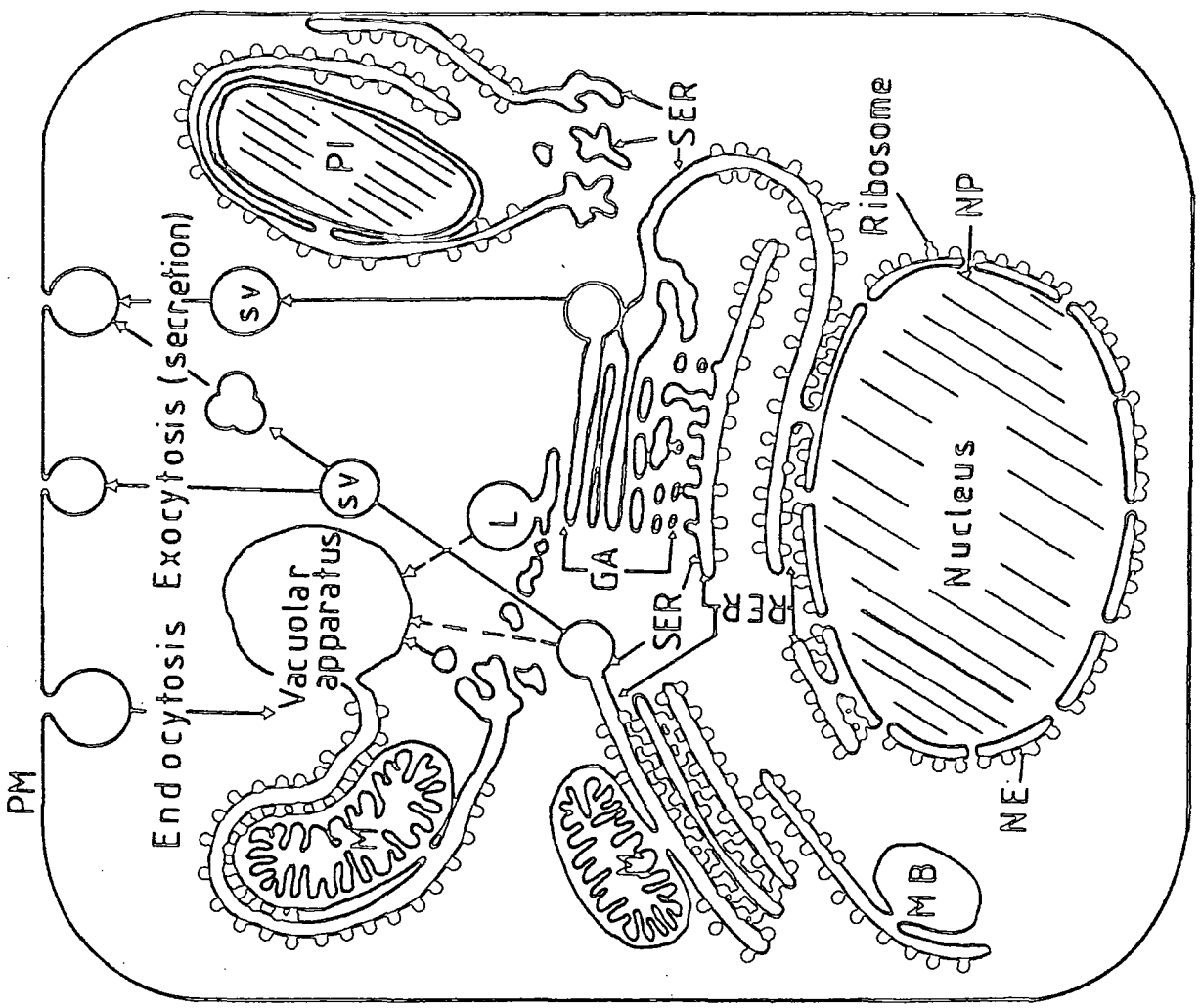
These micrographs clearly show that the yeast cells were transformed with the plasmid pDUB2018, and the cDNA vicilin was expressed to a high degree. The background labelling was very light, showing good hybridisation of vicilin with label. The label was particularly associated with cytoplasmic regions, as expected, of particular interest was its association with membrane structure expected to be involved with the secretory pathway of the vicilin. The secretory pathway in yeast has been discussed at great length in earlier section, and therefore requires no further elaboration in this discussion, but the putative structure observed in this section do require some discussion.

The pathway of secretion is shown in figure 3.4.6 (yeast) and 3.4.7 (higher eukaryotes), the process involves a number of organelles, flowing one to another, until the particular protein is deposited in its ' niche '; i.e. cytoplasm to er to g to vacuole or vesicle or secretion from the cell. The budding cells were selected as they show greater activity, and therefore,

FIGURE 3.4.7

Secretory and Vacuolar protein transport pathways in higher eukaryotes. Nuclear envelope (NE), nuclear pore (NP), Rough and Smooth endoplasmic reticulum (RER,SER), mitochondria (M), golgi apparatus (GA), lyzosome (L), secretory vesicle (SV), plasma membrane (PM).

FIG.3.4.7


PM
Endocytosis
Exocytosis (secretion)
SV
SV
Vacuolar apparatus
L
GA
SER
RER
SER
Ribosome
NP
Nucleus
NE
MB
M
PI

greater movement in the secretory pathway, particularly secretory vesicles to the daughter cell. It was hoped that this would facilitate the study of expression of the vicilin protein, this was realised.

The cDNA-vicilin used in the plasmid pDUB2018 (figure 3.2.5), does not contain a leader sequence, which in the ideal situation is required for early movement in the pathway. The leader sequence is required for positive association and entry into the lumen of the er. So therefore, if this leader sequence is not present, why is the functional protein associated with membrane structures. This may not be the case, as a large proportion of the label is cytoplasmic. However, the micrographs have shown the vicilin to be membrane associated, particularly figure 3.4.4 B. The protein may be translated on ' Free ' ribosomes and then recognised as ' foreign ' and subsequently engulfed by vesicular bodies, which may then move to the vacuole to degrade the protein. Alternatively, the presence of the signal sequence in yeast may not be a prequisite, or may not be as specific in its reqirements as higher eukaryotes. This is considered highly unlikely as there is a wealth of evidence showing the necessity of a signal peptide. Another possibility is that hydrophobic regions in the protein cause it to be associated with membranes.

Many questions arise from the speculation above, these require further study, and particularly, comparison with vicilin having a signal peptide. The sequential movement of this vicilin through the secretory pathway may be further elucidated using such as the *sec* mutants (figure 3.4.6) obtained by

Schekman and his co-workers. The molecular signals that direct vicilin to the vacuole may then be determined.

CHAPTER 4

GENERAL DISCUSSION.

4. GENERAL DISCUSSION.

Due to the time span of this project various aims were not realised. The original aims and strategies can be seen in the particular sections 3.1 to 3.4, but these involved the production of (i) a recombinant genomic vicilin plasmid, (ii) a recombinant vicilin cDNA plasmid, and the analysis of their expression, (iii) a study of transformation efficiencies of these and other plasmids, and (iv) the analysis of expression using electronmicroscopic techniques. Although this project was ambitious for the alloted time span, it was necessary to gain a understanding of the requirements of research, and in doing so the understanding of what may or may not be acheived.

The project itself provided some answers, but more to the point new questions are realised because of these answers. These involve the question of the movement of the vicilin in the cell, from one organelle to another; the role of the leader sequences; the nature of the molecular signals in recognition and translocation of the vicilin, and so on. These answers may be provided by further studies by transforming these recombinant plasmids into the *sec* mutants (figure 3.4.6), which stop the secretory process at various stages in movement of proteins throughout the cell, and viewing with the electronmicroscope. Another way to study this problem may the involvement of pulse-chase experiments, following the vicilin through its sequential movements. The study of clathrin may be very exciting as this may hold the key to specific and non-specific movement in the eukaryotic cell. Clathrin forms ' cage ' structures, in a variety of orientations, and is composed of light and heavy chain regions -

forming polyhedral structures with pentagonal and hexagonal faces. R. Schekman (personal communication) has shown that his *cla1* mutants do not have the heavy chain of clathrin, and supposes it is not necessary for transport, but may allow stabilition of such as the vesicular bodies, dicating its size and shape. Clathrin may stop such as non-specific fusion, and organise certain reactions in the golgi, i.e. α factor precursors. Another approach may be to do a reciprocal study of yeast genes for vacuolar proteins in plant tissue to ascertain if the secretory pathways share some homology ?

The project itself has allowed an understanding of molecular biological techniques used, to study and analyse the recombinant DNA plasmids, and in that sense has been a great success. But the project itself was not finished due to the alocated time.

Recombinant DNA research shows great promise in furthering understanding of yeast and plant biology by making possible the analysis and manipulation of yeast and plant genes not only in the test tube but also in yeast cells. The technological advances have combined to make feasible truly molecular as well as classical genetic manipulation and analysis in yeast. It is hoped that the experimental methologies and approaches given in this study will stimulate further development and exploitation of yeast to answer questions in eukaryotic molecular biology. The opportunity that the yeast system provides for the blending of ideas and methods of classical genetic analysis and modern biochemical and biophysical ideas and methods. Cloning of genes can be acheived in almost any organism and, by itself, is only of limited valve. It is the

ability to return cloned genes (intact or suitably altered) to yeast or higher eukaryotes, either as extra copies or as replacements for the normal form in the normal position in the genome, which makes yeast uniquely suited to this blending of ideas and methods that has been so successful in prokaryotic molecular biology.

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APPENDICES

A

FRAGMENT SIZES (Kb):

λEcoRI	λHindII
23.7	11.25
9.5	4.9
6.6	4.5
4.3	4.2
2.1	2.8
1.9	2.7
0.59	2.55
0.15	2.25
	2.1
	1.65
	1.11
	1.05
	0.705
	0.45
	0.435

B

FRAGMENT SIZES (Kb):

pYSV9 EcoRI
4.2
3.355
2.0
1.032 (this fragment is further cut with HindIII to give a 1.0 and 0.032 Kb fragments)

A

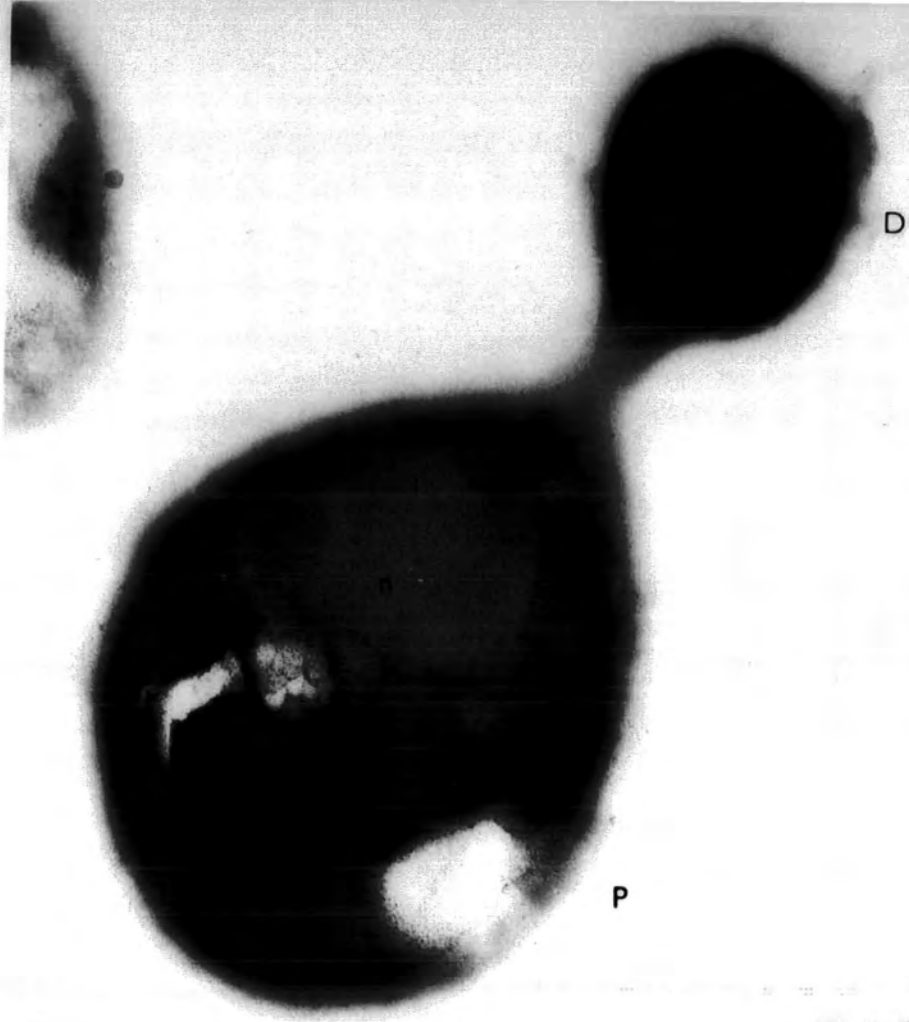
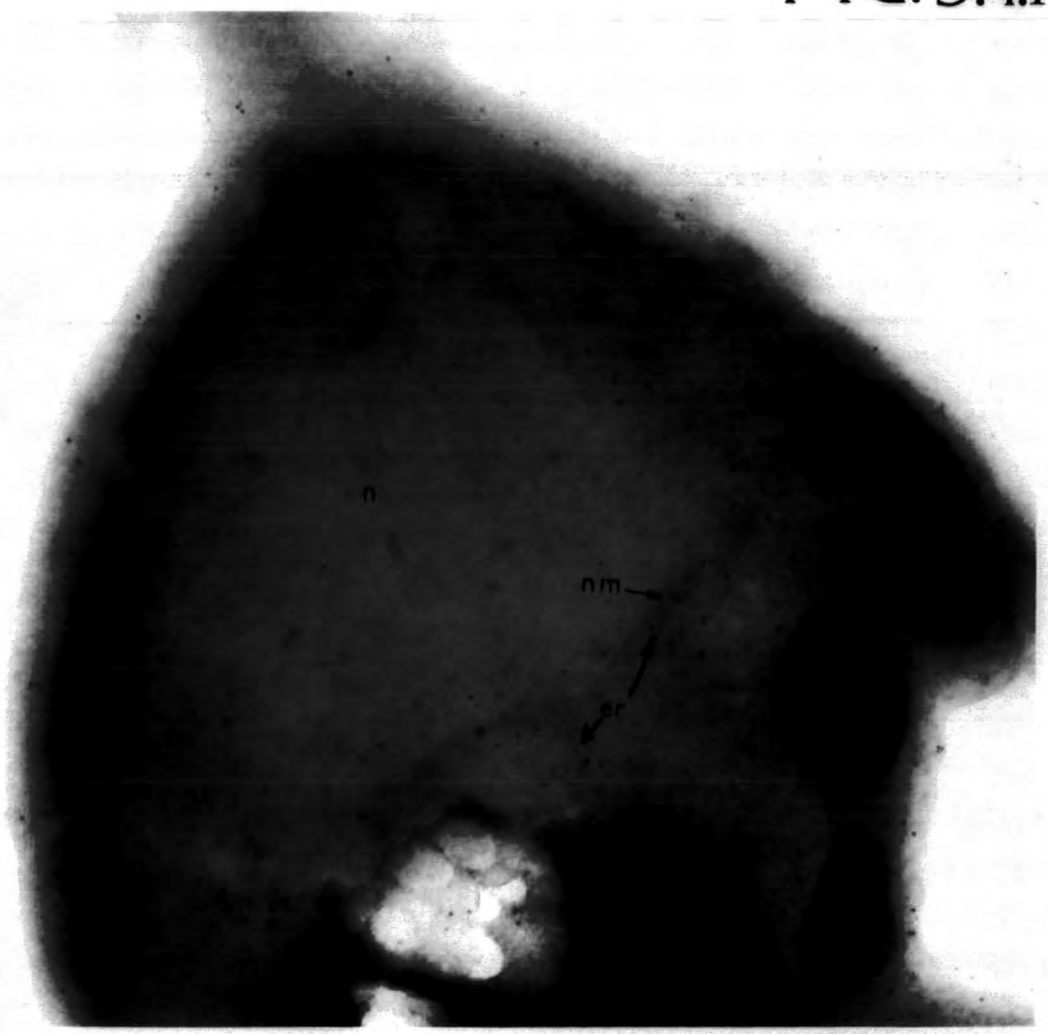


FIG. 3.4.1

B



A

B

FIG. 3.4.2

FIG. 3.4.3

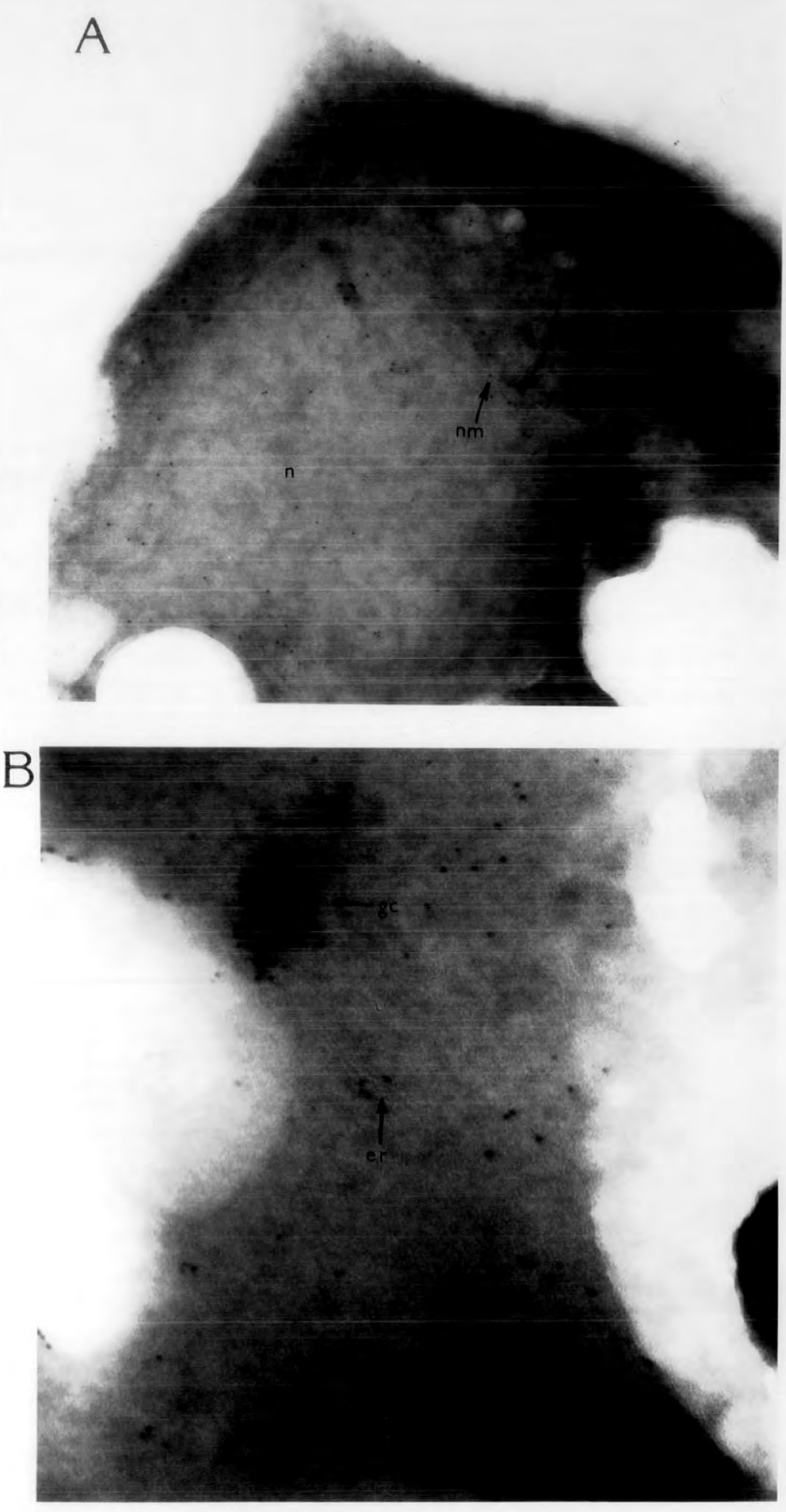


FIG. 3.4.4

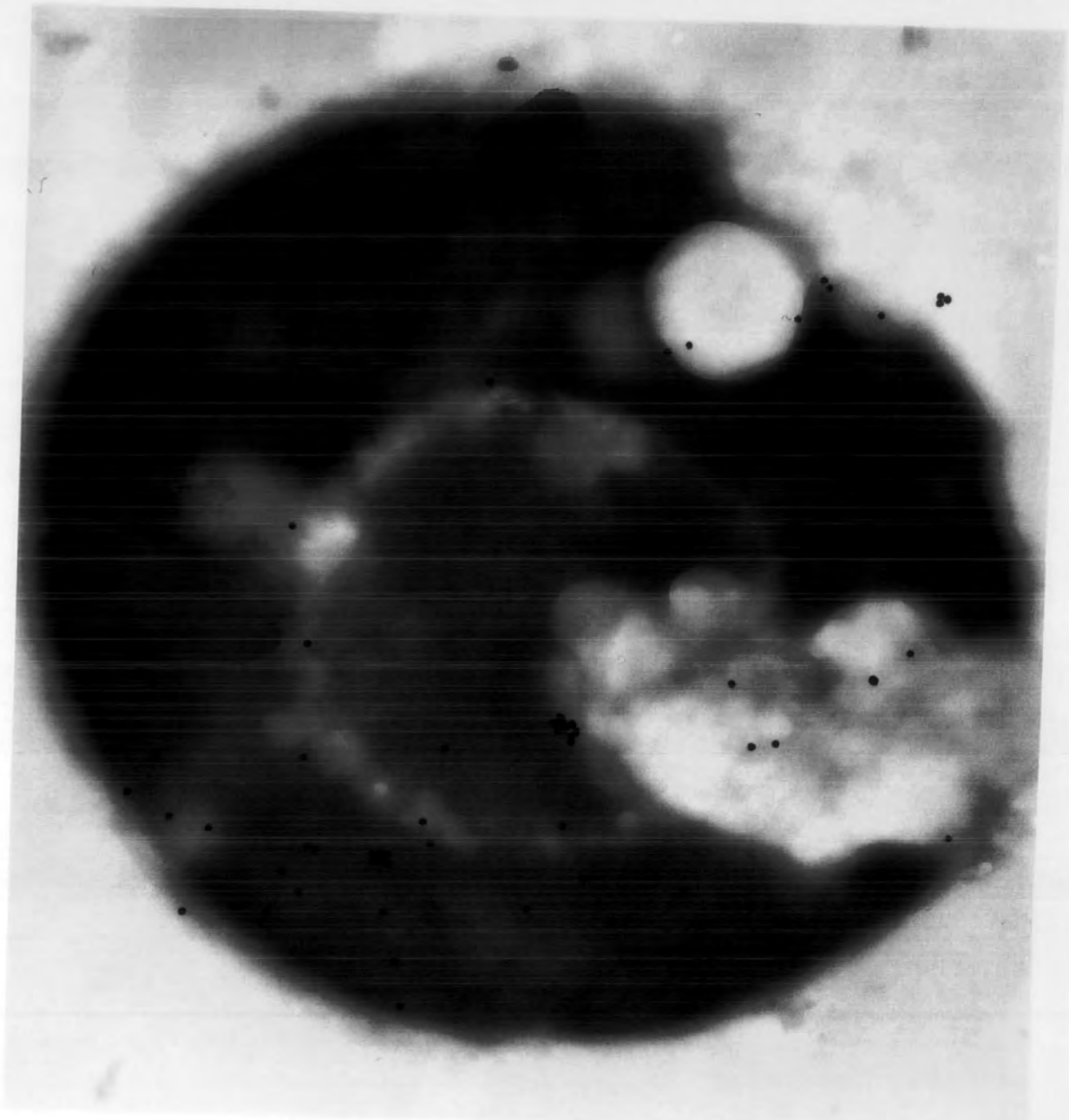


FIG. 3.4.5

The plasmid pDUB2018 contains the cDNA vicilin insert (correct orientation) discussed in section 3.2. The plasmid itself is constructed from the plasmid pMA257 (A. J. Kingsman - unpublished), with a *LEU 2* region, which is defective; 2μ; pBR322; and a Phosphoglycerate Kinase (PGK) promoter, this contains only the 3' and 5' ends with a 14 amino acid region of the PGK N-terminus, and a single BamHI site between the 3' and 5' ends. This allows high copy number in the yeast, and therefore a large amount of the vicilin is produced - this can be observed in all the micrographs recorded (figures 3.4.1 to 3.4.5 using both the 5 and 15nm gold label).

The aim of this part of the project was to compare the results obtained from the other parts - sections 3.1 and 3.2, with the plasmid pDUB2018 results, this was not possible due to the time span of the project, and therefore only the pDUB2018 results can be discussed. It was hoped that the secretion pathway to the yeast vacuole could be shown (section 1.6 - figure 3.4.6 and 3.4.7).

Figure 3.4.1 A shows a budding cell of *S. cerevisiae* at the magnification x19,500. Various organelles and membrane structures can be seen, but at this magnification the gold labelling (black dots) are not easily discernable. Although this figure shows the overall view. Figure 3.4.1 B shows a more magnified view of this yeast, x43,000. The labell (5nm) can mostly be observed in the cytoplasm (c), but can also be seen in the nucleus (n), and can be seen associated with the endoplasmic reticulum (er) and golgi apparatus (g).

Figure 3.4.2 A shows a greater magnified section of figure 3.4.1 (x70,000), this gives a clearer view of the nuclear region; showing black dots associated with the nuclear membrane (nm), the er, and the g. Moving up the section, figure 3.4.2 B (x70,000), shows the dots clearly membrane associated (g), and their association, in the daughter cell, with a possible golgi complex (gc) derived from the paternal cell.

Figure 3.4.3 A and B show daughter cells, B akin to the ones above (x90,000), and A with a magnification x70,000 akin to those in figure 3.4.4. B shows most of the cell; a variety of membrane structures with vicilin can be discerned, corresponding to er, gc and g. A shows putative membrane structures (↓), with possible associated vicilin. The vicilin dots seem to be associated with the darker staining cytoplasm, possibly vesicular.

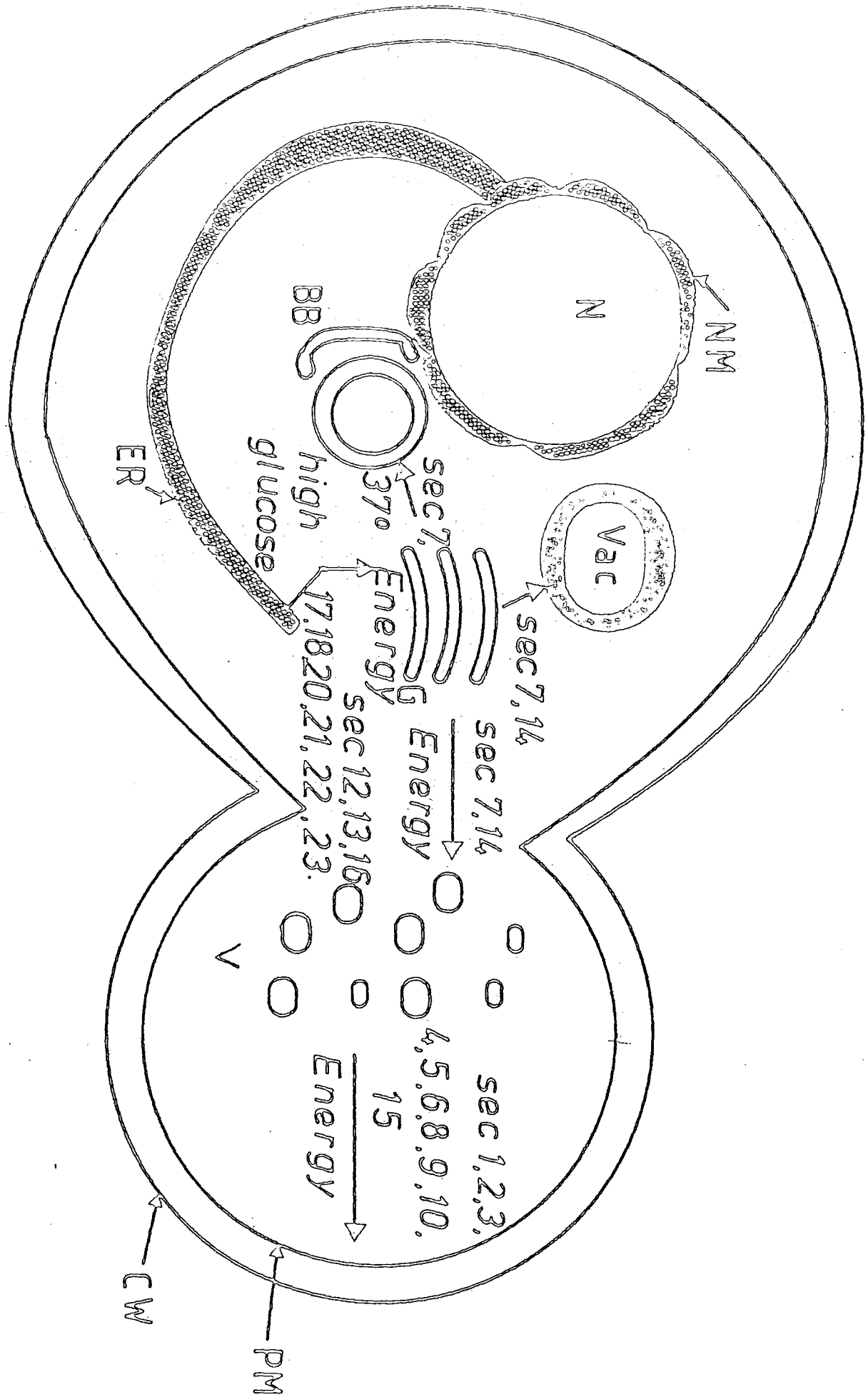
Figure 3.4.4 A shows a similar situation to figure 3.4.1 B, n, nm, and er. But figure 3.4.4 B shows a highly vicilin associated region, this is possibly vesicular (v) or gc in nature. This very important as the gc (Dr. G. Warren - personal communication), which have been reported in higher eukaryotes, may be present in yeast. Warren has proposed that these structures are an intermediate when the cell is duplicating itself. The golgi apparatus is disbanded into this intermediate, allowing movement throughout the cytoplasm, and reforming into the golgi again were required by the cell. It is possible that this structure, found on figure 3.4.4 B, is in the process of disbanding, possibly reappearing in such as the daughter cell. This could possibly show a unproposed form of transport in the yeast cell. Recently Dunphy *et al.* (1986) [25] have stated that

FIGURE 3.4.6

Secretory and Vacuolar protein transport pathways in yeast. The particular *sec* mutants are shown in each step of the pathway.

N = nucleus, NM = nuclear membrane, ER = endoplasmic reticulum, G = golgi apparatus, Vac = vacuole, BB = Berkley body, V = vesicles, PM = plasma membrane, and CW = cell wall.

FIG.3.4.6


NM
N
BB
ER
high glucose
37°
sec 7,
Energy
sec 7,14
Vac
sec 7,14
Energy
sec 12,13,16
17,18,20,21,22,23.
sec 1,2,3,
4,5,6,8,9,10,
15
Energy
CM
PM

yeast and mammals utilise similar cytosolic components to drive protein transport through the golgi apparatus, so it is therefore quite possible this intermediate complex well be found in yeast. Also the vicilin can be seen associated with a double membrane stucture, possibly er or g.

Figure 3.4.5 simply shows the difference between the 5nm, seen above, to the 15nm gold labelling. Although the section does not show organelle structure to any degree, it does show that the 5nm labelling is more specific and one is capable to discern a higher degree of compartmentalisation within the cells.

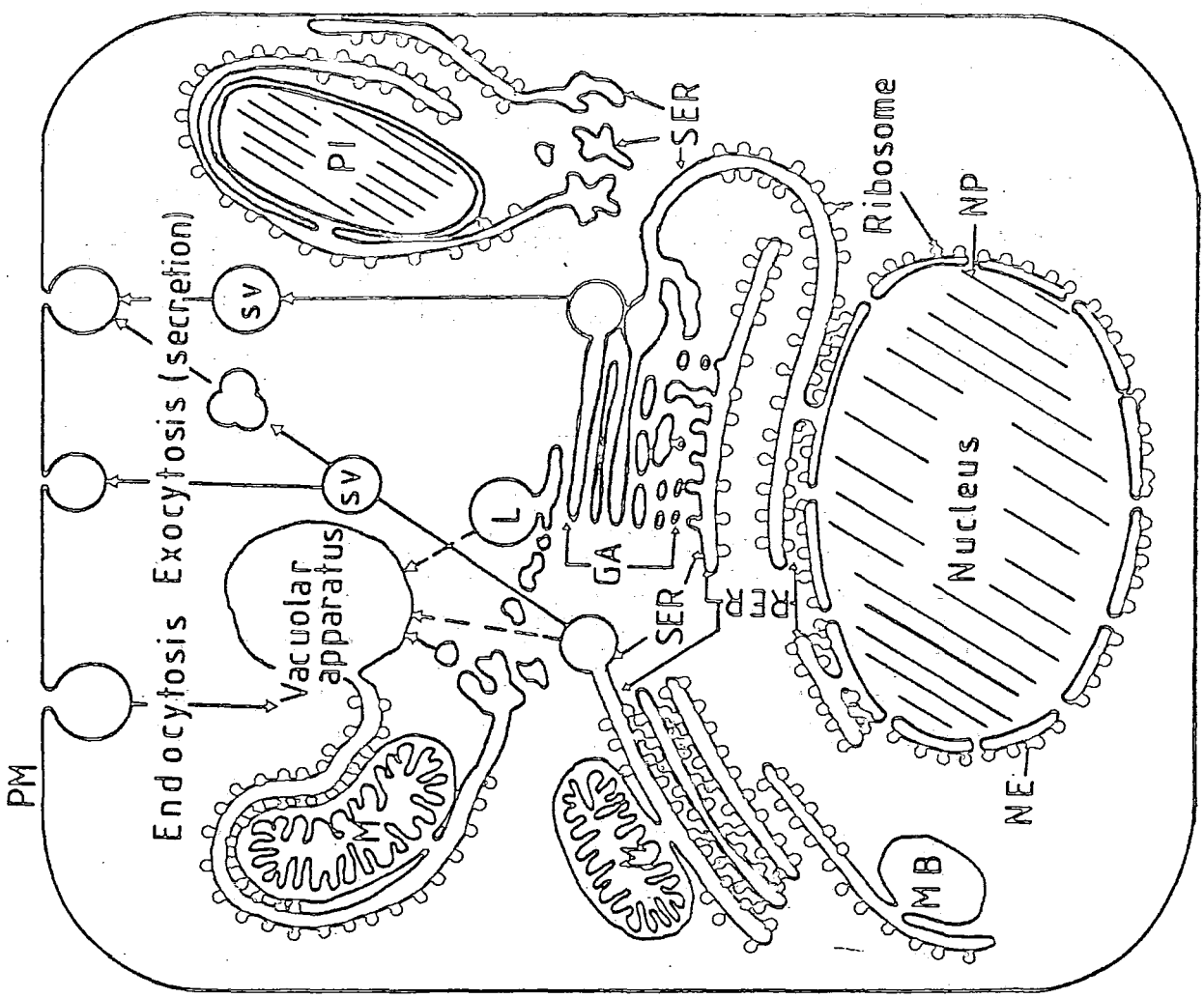
These micrographs clearly show that the yeast cells were transformed with the plasmid pDUB2018, and the cDNA vicilin was expressed to a high degree. The background labelling was very light, showing good hybridisation of vicilin with label. The label was particularly associated with cytoplasmic regions, as expected, of particular interest was its association with membrane structure expected to be involved with the secretory pathway of the vicilin. The secretory pathway in yeast has been discussed at great length in earlier section, and therefore requires no further elaboration in this discussion, but the putative structure observed in this section do require some discussion.

The pathway of secretion is shown in figure 3.4.6 (yeast) and 3.4.7 (higher eukaryotes), the process involves a number of organelles, flowing one to another, until the particular protein is deposited in its ' niche '; i.e. cytoplasm to er to g to vacuole or vesicle or secretion from the cell. The budding cells were selected as they show greater activity, and therefore,

FIGURE 3.4.7

Secretory and Vacuolar protein transport pathways in higher eukaryotes. Nuclear envelope (NE), nuclear pore (NP), Rough and Smooth endoplasmic reticulum (RER,SER), mitochondria (M), golgi apparatus (GA), lyzosome (L), secretory vesicle (SV), plasma membrane (PM).

FIG.3.4.7


PM
Endocytosis
Exocytosis (secretion)
sv
sv
sv
Vacuolar apparatus
L
GA
SER
RER
SER
PI
Ribosome
NP
Nucleus
NE
MB

greater movement in the secretory pathway, particularly secretory vesicles to the daughter cell. It was hoped that this would facilitate the study of expression of the vicilin protein, this was realised.

The cDNA vicilin used in the plasmid pDUB2018 (figure 3.2.5), does not contain a leader sequence, which in the ideal situation is required for early movement in the pathway. The leader sequence is required for positive association and entry into the lumen of the er. So therefore, if this leader sequence is not present, why is the functional protein associated with membrane structures. This may not be the case, as a large proportion of the label is cytoplasmic. However, the micrographs have shown the vicilin to be membrane associated, particularly figure 3.4.4 B. The protein may be translated on ' Free ' ribosomes and then recognised as ' foreign ' and subsequently engulfed by vesicular bodies, which may then move to the vacuole to degrade the protein. Alternatively, the presence of the signal sequence in yeast may not be a prequisite, or may not be as specific in its reqirements as higher eukaryotes. This is considered highly unlikely as there is a wealth of evidence showing the necessity of a signal peptide. Another possibility is that hydrophobic regions in the protein cause it to be associated with membranes.

Many questions arise from the speculation above, these require further study, and particularly, comparison with vicilin having a signal peptide. The sequential movement of this vicilin through the secretory pathway may be further elucidated using such as the *sec* mutants (figure 3.4.6) obtained by

Schekman and his co-workers. The molecular signals that direct vicilin to the vacuole may then be determined.

CHAPTER 4

GENERAL DISCUSSION.

4. GENERAL DISCUSSION.

Due to the time span of this project various aims were not realised. The original aims and strategies can be seen in the particular sections 3.1 to 3.4, but these involved the production of (i) a recombinant genomic vicilin plasmid, (ii) a recombinant vicilin cDNA plasmid, and the analysis of their expression, (iii) a study of transformation efficiencies of these and other plasmids, and (iv) the analysis of expression using electronmicroscopic techniques. Although this project was ambitious for the alloted time span, it was necessary to gain a understanding of the requirements of research, and in doing so the understanding of what may or may not be acheived.

The project itself provided some answers, but more to the point new questions are realised because of these answers. These involve the question of the movement of the vicilin in the cell, from one organelle to another; the role of the leader sequences; the nature of the molecular signals in recognition and translocation of the vicilin, and so on. These answers may be provided by further studies by transforming these recombinant plasmids into the *sec* mutants (figure 3.4.6), which stop the secretory process at various stages in movement of proteins throughout the cell, and viewing with the electronmicroscope. Another way to study this problem may the involvement of pulse-chase experiments, following the vicilin through its sequential movements. The study of clathrin may be very exciting as this may hold the key to specific and non-specific movement in the eukaryotic cell. Clathrin forms ' cage ' structures, in a variety of orientations, and is composed of light and heavy chain regions -

forming polyhedral structures with pentagonal and hexagonal faces. R. Schekman (personal communication) has shown that his *cla1* mutants do not have the heavy chain of clathrin, and supposes it is not necessary for transport, but may allow stabilition of such as the vesicular bodies, dicating its size and shape. Clathrin may stop such as non-specific fusion, and organise certain reactions in the golgi, i.e. α factor precursors. Another approach may be to do a reciprocal study of yeast genes for vacuolar proteins in plant tissue to ascertain if the secretory pathways share some homology ?

The project itself has allowed an understanding of molecular biological techniques used, to study and analyse the recombinant DNA plasmids, and in that sense has been a great success. But the project itself was not finished due to the alocated time.

Recombinant DNA research shows great promise in furthering understanding of yeast and plant biology by making possible the analysis and manipulation of yeast and plant genes not only in the test tube but also in yeast cells. The technological advances have combined to make feasible truly molecular as well as classical genetic manipulation and analysis in yeast. It is hoped that the experimental methologies and approaches given in this study will stimulate further development and exploitation of yeast to answer questions in eukaryotic molecular biology. The opportunity that the yeast system provides for the blending of ideas and methods of classical genetic analysis and modern biochemical and biophysical ideas and methods. Cloning of genes can be acheived in almost any organism and, by itself, is only of limited valve. It is the

ability to return cloned genes (intact or suitably altered) to yeast or higher eukaryotes, either as extra copies or as replacements for the normal form in the normal position in the genome, which makes yeast uniquely suited to this blending of ideas and methods that has been so successful in prokaryotic molecular biology.

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APPENDICES

A

FRAGMENT SIZES (Kb):

λEcoRI	λHindII
23.7	11.25
9.5	4.9
6.6	4.5
4.3	4.2
2.1	2.8
1.9	2.7
0.59	2.55
0.15	2.25
	2.1
	1.65
	1.11
	1.05
	0.705
	0.45
	0.435

B

FRAGMENT SIZES (Kb):

pYSV9 EcoRI
4.2
3.355
2.0
1.032 (this fragment is further cut with HindIII to give a 1.0 and 0.032 Kb fragments)